

Novel Aspects of Growth Hormone (GH) Autoregulation: GH-Induced GH Gene Expression in Grass Carp Pituitary Cells through Autocrine/Paracrine Mechanisms

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GH feedback on its own secretion at the pituitary level has been previously reported, but the mechanisms involved have not been elucidated. Here we examined the autocrine/paracrine effects of GH on GH synthesis using grass carp pituitary cells as a cell model. GH receptors were identified in carp somatotrophs, and their activation by exogenous GH increased steady-state GH mRNA levels and GH production. Removal of endogenous GH by immunoneutralization using GH antiserum inhibited basal as well as stimulated GH mRNA expression induced by GH-releasing factors in fish, including GnRH, apomorphine, and pituitary adenylate cyclase-activating polypeptide-38. Cytosolic mature GH mRNA levels were elevated by GH treatment and reduced by GH antiserum,

whereas nuclear GH primary transcripts were almost undetectable after GH immunoneutralization. Inhibition of Janus kinase-2 (JAK₂), phosphoinositide 3-kinase, and MAPK also abolished GH-induced steady-state GH mRNA expression. GH immunoneutralization in pituitary cells pretreated with actinomycin D induced a marked decrease in the half-life of GH mRNA, indicating that the clearance of GH transcripts could be enhanced by removing endogenous GH. These results provide evidence that GH can serve as a novel intrapituitary autocrine/paracrine factor maintaining GH gene expression in somatotrophs, and this action is mediated by JAK₂/MAPK and JAK₂/phosphoinositide 3-kinase cascades coupled to GH receptors. (*Endocrinology* 145: 4615–4628, 2004)

IN MAMMALS, GH release from somatotrophs is under the control of hypothalamic regulators, hormones released from target organs/peripheral tissues, feedback regulation by GH itself, and local interactions of autocrine/paracrine factors within the pituitary (1–4). Regarding GH feedback, indirect actions via IGF production in the liver constitute a long-loop feedback on GH secretion and synthesis (5). GH can penetrate the blood-brain barrier by receptor-mediated transcytosis at the choroid plexus (6). Subsequent activation of GH receptors in the hypothalamus alters the functionality of arcuate and periventricular nuclei to induce somatostatin and inhibit GHRH release into hypophyseal portal blood (7), probably via neuropeptide Y interneurons (8). This short-loop feedback of GH can be reduced by central administration of GH antagonists (9) or brain infusion of an antisense against GH receptor mRNA (10). Besides, an ultrashort feedback of GH by acting directly at the pituitary level through autocrine/paracrine mechanisms has been proposed. This ultrashort component is sup-

ported by the findings that GH treatment inhibits GH release in bovine pituitary cells (11). In the rat, GH receptors are ubiquitously expressed in the pituitary (12). Furthermore, transgenic mice with GH receptor knockout or expression of a GH antagonist exhibit histological features in somatotrophs typical of secretory hyperactivity (13). In lower vertebrates, *e.g.* fish, ectopic expression of GH in transgenic salmon inhibits GH mRNA expression and reduces the size of the pituitary (14). At the pituitary level, ultrashort feedback of GH release has been inferred as treatment of intact trout pituitaries with ovine GH and can also suppress GH secretion (15).

However, there are reports with results at variance with the ultrashort feedback model. For example, GH treatment does not affect GH release in rat pituitary cells (16) and enriched rat somatotrophs (17). Pituitary transplanted under the renal capsule has been used as a model to study GH release in the absence of hypothalamic influences (18) and has been recently adopted as a means to prolong the survival of cotransplanted islets for diabetic therapy (19). In these *in vivo* studies, a normal range of GH levels in blood can be detected after grafting of pituitary fragments into the renal capsule. In static cultures of pituitary cells, a progressive increase in GH levels is commonly observed for days to weeks, even in the absence of GH secretagogues (for literature review, see Ref. 20). If ultrashort feedback indeed is a part of the physiological mechanisms regulating GH secretion, it will be rather difficult to interpret the results of these *in vivo* and *in vitro* studies because local actions of endogenous GH should have inhibited basal GH release. Recently

Abbreviations: [Ca²⁺]_i, Intracellular Ca²⁺ concentration; % Control, percentage of the corresponding mean value in the control group; DIG, digoxigenin; GTH, gonadotropin; IHC, immunohistochemical; IRS, insulin receptor substrate; JAK₂, Janus kinase-2; LCM, laser capture microdissection; NIL, neurointermediate lobe; NRS, normal rabbit serum; PACAP, pituitary adenylate cyclase-activating polypeptide; PI3K, phosphoinositide 3-kinase; PRL, prolactin; RPA, ribonuclease protection assay; RPD, rostral pars distalis; RT, reverse transcription; STAT, signal transducer and activator of transcription.

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there has been increasing evidence for local interactions of hormonal factors in the pituitary to modulate pituitary functions (2). The paradoxical findings regarding ultrashort feedback may suggest the possible existence of an autocrine/paracrine component at the pituitary level to maintain spontaneous GH secretion irrespective of the potential autoinhibition of GH release.

Because there have been no previous studies on the autocrine/paracrine actions of GH on GH gene expression, it is unclear whether the intrapituitary feedback of GH also plays a role in GH synthesis. Although GH is known to exert its biological actions by coupling to the Janus kinase-2 (JAK₂)/signal transducer and activator of transcription (STAT), JAK₂/MAPK, and/or JAK₂/insulin receptor substrate (IRS)/phosphoinositide 3-kinase (PI3K) pathways (21), the postreceptor signaling mechanisms responsible for GH ultrashort feedback are still unknown. To address these questions, grass carp pituitary cells were used as a cell model to study the functional relevance, molecular mechanisms, and signal transduction of GH gene expression in response to GH stimulation at the pituitary level. In this study, we have shown for the first time that endogenous GH acts as a novel autocrine/paracrine factor in the pituitary to maintain GH release, GH gene expression, and somatotroph sensitivity to stimulation by GH-releasing factors. GH-induced GH mRNA expression is mediated by improving GH transcript stability and GH gene transcription. Apparently the stimulatory action of GH on GH gene expression is dependent on the functional coupling of JAK₂, PI3K, and MAPK to pituitary GH receptors.

Materials and Methods

Animals

One-year-old (1+) grass carps (*Ctenopharyngodon idellus*) with body weight about 1.5–2.0 kg and gonadosomatic index 0.2% or less were purchased from local wholesale markets and kept in well-aerated 200-liter aquaria under 12-h light, 12-h dark photoperiod at 18 ± 2 C for at least 6 d before pituitary cell preparation. Fish were killed by anesthesia in MS222 (Sigma, St. Louis, MO) followed by spinosectomy according to the regulations of animal use at the University of Hong Kong.

Reagents and test substances

Porcine GH and apomorphine were obtained from Sigma, and salmon GnRH and ovine pituitary adenylate cyclase-activating polypeptide-38 (PACAP) were purchased from Phoenix Pharmaceuticals (Belmont, CA). Sea bream GH was acquired from GroPep Pty. Ltd. (North Adelaide, Australia). Actinomycin D, PD98059, SB202190, AG490, and wortmannin were obtained from Calbiochem (San Diego, CA). Peptide hormones, including GH, GnRH, and PACAP, were prepared in 1 mM stocks in double-distilled deionized water and the pharmacological agents, including wortmannin, PD98059, SB202190, and AG490, were dissolved in dimethylsulfoxide to form 10 mM stocks. Stock solutions were diluted to appropriate concentrations with culture medium before adding to pituitary cell cultures. The final levels of dimethylsulfoxide were always 0.1% or less and did not alter GH secretion and GH mRNA expression.

Measurement of GH production

Grass carp pituitary cells were prepared by trypsin/DNase digestion method as described previously (22) and cultured in 24-well plates (Costar, Corning Inc., Corning, NY) at a seeding density of approximately 0.75×10^6 cells/ml per well in carp MEM [MEM Eagle with 26 mM NaHCO₃, 25 mM HEPES, and 1% antibiotic-antimycotic (pH 7.7)]

with 5% fetal bovine serum. After culturing for 18 h, culture medium was replaced with serum-free carp MEM, and drug treatment was initiated for the duration as indicated in individual studies. After that, culture medium was collected for the measurement of GH release, and protein extract was prepared from pituitary cells for quantitation of cellular GH contents. GH levels in these samples were quantified by a RIA previously validated for grass carp GH (23), and GH production was defined as the sum of GH content and GH release. The detection limit of the RIA system was 0.4 ± 0.1 ng GH/ml, whereas the intra- and interassay coefficients of variation were found to be 5.06 ± 0.78 and $6.3 \pm 1.36\%$, respectively ($n = 16$).

Measurement of steady-state GH mRNA expression

Pituitary cells were cultured in 24-well plates (Costar) at a seeding density of approximately 2.5×10^6 cells/ml per well. After drug treatment, culture medium was aspirated and pituitary cells were dissolved in TRIZOL (Gibco, Gaithersburg, MD). Total RNA (~2.8 μg/well) was isolated by phase separation followed by precipitation of the aqueous phase using isopropanol. Genomic DNA in the organic phase was precipitated using absolute ethanol, dissolved in 8 mM NaOH, and quantified by reading the absorbance at 260 nm. Total RNA obtained was heat denatured at 70 C and vacuum blotted onto a positively charged nylon membrane using a Bio-Dot SF microfiltration unit (Bio-Rad Laboratories, Hercules, CA). The membrane was then UV cross-linked, prehybridized for 3 h, and hybridized overnight at 42 C with a digoxigenin (DIG)-labeled cDNA probe for grass carp GH mRNA. The probe was labeled with a DIG probe synthesis kit (Roche, Stockholm, Sweden) using primers flanking position 75 and 444 of grass carp GH cDNA (GenBank no. M27094). Hybridization signals of RNA samples prepared from individual wells were visualized using a DIG luminescent detection kit (Roche) and quantified using an IC440 digital science image station (Eastman Kodak, New Haven, CT). In these experiments, parallel probing of the duplicated membrane using a DIG-labeled probe for grass carp 18S RNA was used as an internal control. To confirm the specificity of GH immunoneutralization on GH mRNA expression, slot blot of gonadotropin (GTH) α-subunit mRNA was also performed using a DIG-labeled probe flanking position 82 to 344 of grass carp GTHα cDNA (GenBank no. X61050). To test whether treatment with exogenous GH and GH antiserum could alter the form(s) and/or size of GH transcripts, Northern blot was conducted using mRNA prepared from grass carp pituitary cells. In this case, total RNA was obtained from pituitary cells cultured at a seeding density of 15×10^6 cells/10 ml per dish. After that, mRNA was isolated using a Poly-A Tract mRNA isolation system III (Promega, Madison, WI). These mRNA samples were resolved in 1% agarose gel with 0.22 M formaldehyde and transferred onto positively charged nylon membrane. Hybridization signals for GH mRNA were developed and analyzed as described in the preceding section for slot blot assays.

Immunohistochemical staining of pituitary cells

Pituitaries were freshly excised from grass carps, fixed in Bouin's fixative, embedded in paraffin wax, and sectioned into 5 μm in thickness according to standard procedures. For immunostaining of dispersed pituitary cells, cytospin preparations were prepared using an Autospin CF-120 centrifuge (Sakura Fine Technical, Nagand, Japan) and fixed in Bouin's fixative. Immunohistochemical (IHC) staining was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Antisera for carp GH and prolactin (PRL) (gift from Dr. R. E. Peter, University of Alberta, Alberta, Canada; polyclonal antisera raised in rabbit) were used at 1:1000 to match with the effective doses of antiserum used in GH immunoneutralization.

RT-PCR of GH receptor in laser capture microdissection (LCM)-isolated somatotrophs

Cytospin preparation of grass carp pituitary cells (~ 5×10^4 cells/slide) was prepared and subjected to immunostaining with GH antiserum (1:8000 dilution) using the Vectastain ABC kit (Vector). After staining, pituitary cells were dehydrated in ethanol, cleared by xylene

treatment, and air dried before LCM. Immunoidentified somatotrophs were isolated with Capsure HS LCM Caps using a PixCell II laser capture microdissection system (Arcturus Engineering Inc., Mountain View, CA). About 50 somatotrophs were captured on individual caps, and total RNA was extracted using TRIZOL. After DNase I digestion and reverse transcription (RT), PCR was conducted using primers covering position 839 and 1097 of grass carp GH receptor cDNA (GenBank no. AY283778), and the authenticity of PCR product (559 bp) was then confirmed by Southern blot (data not shown). In these experiments, RT-PCR of β -actin mRNA was used as an internal control.

Measurement of intracellular Ca^{2+} concentration $[(Ca^{2+})_i]$ in enriched somatotrophs

Enriched somatotrophs were prepared from mixed populations of grass carp pituitary cells by discontinuous density gradient centrifugation (24). These cells were preloaded with Indo-1/AM ($10 \mu\text{M}$; Molecular Probes, Eugene, OR) in HEPES-buffered saline [150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.7)] with 0.1% BSA for 60 min at 28 C. After dye loading, somatotrophs were resuspended in BSA-free HEPES-buffered saline and measurement of $[Ca^{2+}]_i$ was conducted in a 4500 fluorescence spectrophotometer (Hitachi, Indianapolis, IN) with excitation and emission wavelengths at 329 and 405 nm, respectively. In this study, $[Ca^{2+}]_i$ was calibrated using the cell lysis method as described previously (25).

Western blot of pituitary cells after GH immunoneutralization

To examine the effects of GH immunoneutralization on basal GH release and cellular GH contents, Western blot was conducted to avoid possible interference of adding antiserum to cell cultures on subsequent GH RIA. In this case, pituitary cells were either incubated with increasing levels of GH antiserum (raised in rabbit) for 48 h or exposed to GH antiserum (1:2500) for 2, 24, and 48 h, respectively. After that, culture medium was harvested for quantitation of GH release, and cell lysate was prepared from pituitary cells in PBS by three cycles of repeated freezing and thawing. These protein samples were then size fractionated by SDS-PAGE in duplicate in 15% gels under denaturing conditions. One of the gels was used for Coomassie blue staining to reveal protein bands, and the other one was transblotted onto an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked with 2% dried milk for 15 h, incubated with a monoclonal antibody for grass carp GH (1:4000; raised in mouse) for 1.5 h and exposed to horseradish peroxidase-conjugated goat antimouse IgG (Bio-Rad; 1:5000) with SuperSignal West Pico (Pierce, Rockford, IL) as the substrate.

Ribonuclease protection assay for GH primary transcript

For simultaneous monitoring of cytosolic mature GH mRNA and nuclear GH primary transcript, pituitary cells were cultured in 12-well plates (Costar) at a density of approximately 5×10^6 cells/2 ml per well. After drug treatment, pituitary cells were dissolved in an ice-cold lysis solution composed of 10 mM Tris buffer (pH 7.4) with 0.3 M sucrose, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40, and 0.25% sodium deoxycholate. The cell lysate was gently layered on the top of a cushion buffer [0.4 M sucrose and 1.5 mM $MgCl_2$ in Tris buffer (pH 7.4)] and centrifuged at 3300 rpm for 10 min at 4 C. The upper layer with the cytosolic fraction was digested with proteinase K (0.1 mg/ml, Sigma) at 45 C for 1 h. The cushion layer was vortexed vigorously to disrupt the nuclear fraction and exposed to sequential digestion with DNase I (166.6 U/ml, Gibco) for 15 min at 37 C followed by 2 h digestion at 45 C with proteinase K (0.2 mg/ml). After that, RNA from these two layers was purified by phenol/chloroform extraction and subjected to ribonuclease protection assay (RPA) using an Ambion RPA II kit (Ambion Inc., Austin, TX). A DNA fragment with 120 bp exon II and 87 bp intron II of grass carp GH gene (position 348–557; GenBank no. X60419) was used to produce ^{32}P -labeled antisense riboprobe by *in vitro* transcription. Sense-strand cRNAs were also prepared to serve as the standards for mature GH mRNA and GH primary transcript. In this assay, hybridization of riboprobe with mature GH mRNA and GH primary transcript could yield protected fragments of 124 and 207 bp, respectively. ^{32}P radioactivity of these protected frag-

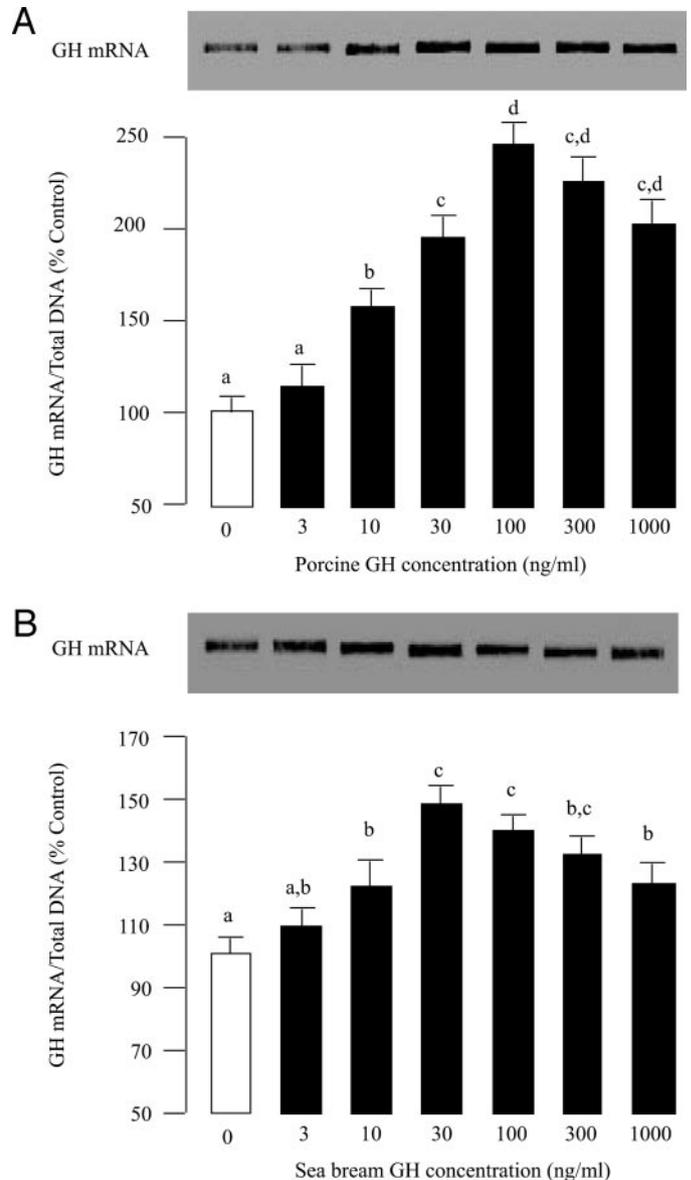


FIG. 1. Effects of GH treatment on GH mRNA expression in grass carp pituitary cells. A, Pituitary cells were exposed to increasing doses of porcine GH for 48 h under static incubation conditions. B, Parallel experiment using increasing doses of sea bream GH as the stimulant. After drug treatment, total RNA was isolated for GH mRNA measurement as described in *Materials and Methods*. GH mRNA data are expressed as mean \pm SEM ($n = 6$ wells), and treatment groups denoted by different letters represent significant difference at $P < 0.05$. Representative results of slot blot are also included for individual experiments.

ments was quantified using a STORM 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Real-time PCR measurement of mature GH mRNA and GH primary transcripts

Pituitary cells were cultured in 12-well plates (Costar) at a seeding density of approximately 5×10^6 cells/2 ml per well. After treatment with GH antiserum, pituitary cells were dissolved in TRIZOL, and total RNA isolated was treated with DNase I. After that, RT was carried out and RT samples were subjected to quantitative PCR using a RotorGene 2000 real-time amplification system (Corbett Research, Eight Mile Plains,

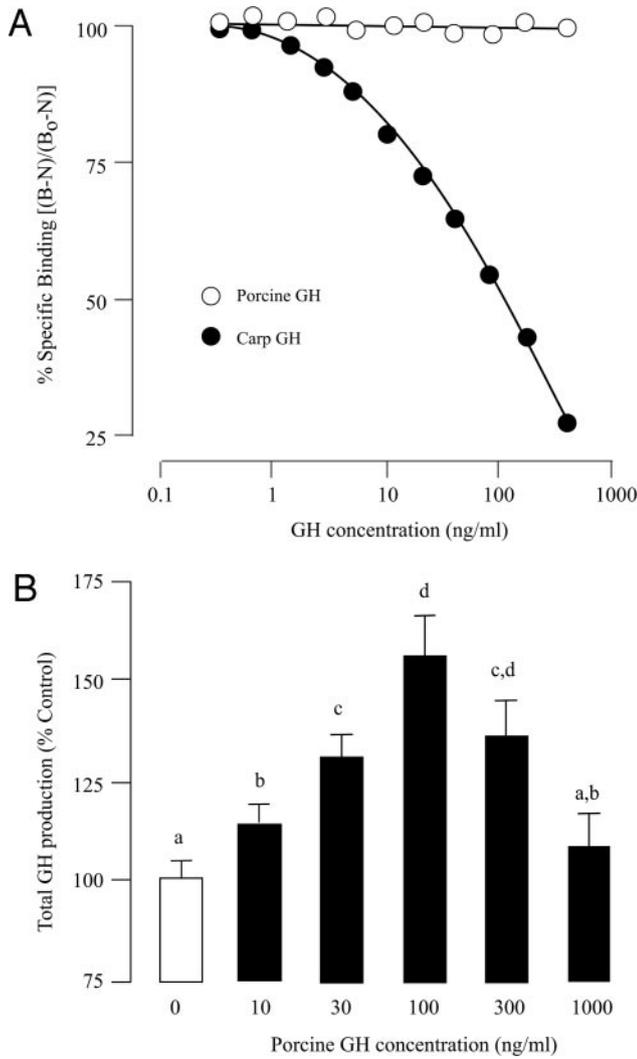


FIG. 2. Effects of GH treatment on GH production in grass carp pituitary cells. **A**, Cross-reactivity of porcine GH in the RIA for grass carp GH. GH antiserum (1:38,000) was incubated with ¹²⁵I-labeled GH tracer (~0.2 μCi/tube) in the presence of increasing levels of porcine GH and carp GH. Separation of bound and free tracer in GH RIA was conducted using the double-antibody precipitation method. **B**, Effects of porcine GH on GH production in grass carp pituitary cells. Pituitary cells were incubated with increasing doses of porcine GH for 48 h. GH data presented are expressed as mean ± SEM (n = 8 wells) and treatment groups denoted by *different letters* represent significant difference at *P* < 0.05.

New South Wales, Australia). PCR was conducted using a LightCycler-DNA master SYBR Green I kit (Roche), and melting analysis was routinely performed to check for the identity of PCR product. Real-time PCR of mature GH mRNA was conducted in RT samples at 1:200 dilution with primers flanking position 75 and 396 of grass carp GH cDNA. These primers selectively produced a 252-bp PCR product with melting temperature at 92.2 C for cytosolic mature GH mRNA. PCR of GH primary transcript was conducted in undiluted RT samples with primers covering position 348 and 557 of grass carp GH gene. These primers were designed to cover the junction between exon II and intron II and were shown to be ineffective in PCR amplification of mature GH mRNA. Based on our validation, these primers could selectively amplify a 210-bp PCR product with melting temperature at 91.4 C for nuclear GH primary transcripts. In this study, serial dilutions of plasmid DNA containing grass carp GH cDNA and full-length GH gene were used as the standards for real-time PCR of mature GH mRNA and GH primary transcripts, respectively.

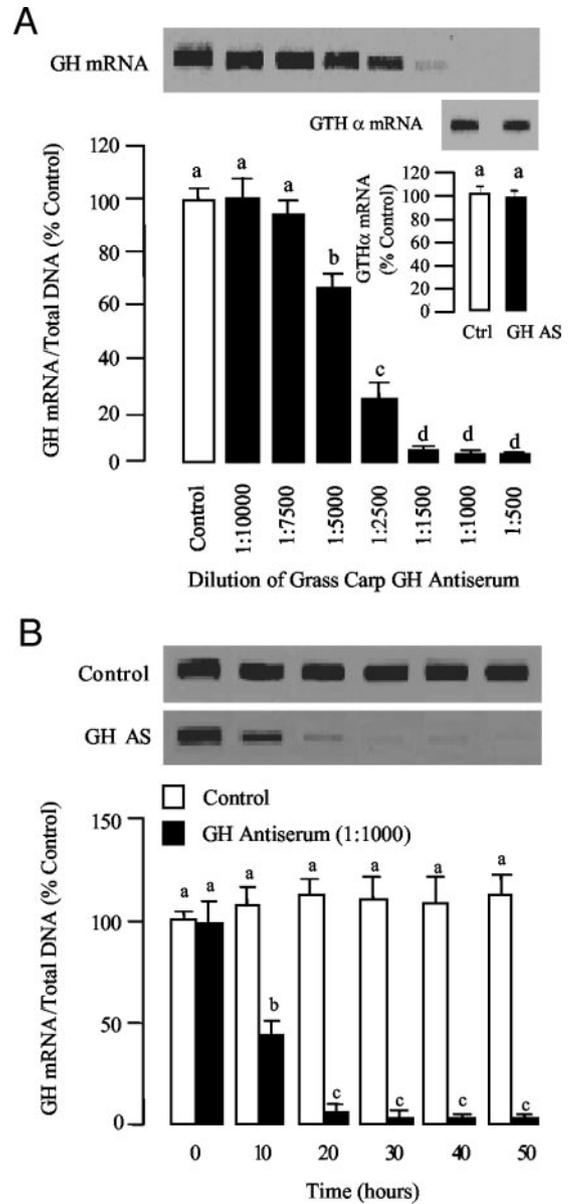


FIG. 3. Effects of GH immunoneutralization on GH mRNA expression. **A**, Dose dependence of GH immunoneutralization on GH mRNA expression was tested by exposing pituitary cells to increasing levels of GH antiserum (GH AS) for 48 h under static incubation. The effect of 48-h incubation with GH AS (1:2500) on GTHα mRNA expression was also examined in carp pituitary cells to serve as a negative control (*small inset*). **B**, Time course of GH immunoneutralization was studied by incubating pituitary cells with GH antiserum (1:1000) for the duration as indicated. GH mRNA data are expressed as mean ± SEM (n = 6 wells), and treatment groups denoted by *different letters* represent significant difference at *P* < 0.05. Representative results of slot blot are also included for individual experiments.

Data transformation and statistics

GH data (in nanograms per million cells) were transformed into a percentage of the corresponding mean value in the control group (as % Control). This normalization was conducted to allow for pooling of data from separate experiments without increasing the overall variability of final results. GH mRNA levels from individual wells were measured in terms of arbitrary density units and normalized against DNA content of the same sample to adjust for potential variations in cell number between wells. In these experiments, no significant changes in 18S RNA levels

were noted (data not shown), and GH mRNA data were simply transformed into % Control for statistical analysis. For $[Ca^{2+}]_i$ measurement, fluorescence data (in arbitrary fluorescence units) were transformed into Ca^{2+} concentrations using the single-wavelength calibration equation: $[Ca^{2+}]_i = K_d \times (F - F_{min}) / (F_{max} - F)$, where K_d is the dissociation constant for Indo-1, F_{min} is basal fluorescence in the absence of Ca^{2+} , F_{max} is the maximal fluorescence under saturating Ca^{2+} , and F is the fluorescence at any unknown Ca^{2+} level. F_{min} and F_{max} were determined empirically for individual dye loading by exposing pituitary cells to sequential treatment of EGTA (4 mM), digitonin (10 mg/ml), and $CaCl_2$ (25 mM). In the case of real-time PCR, standard curves with a dynamic range of 10^5 or more and a correlation coefficient of 0.95 or more were used for data calibration. Levels of mature GH mRNA and GH primary transcript were expressed as femtomoles per million cells and attomoles per million cells, respectively. Data presented in this study were analyzed using Student's *t* test or ANOVA followed by Fisher's least significance difference test. Differences were considered significant at $P < 0.05$.

Results

GH-induced GH mRNA expression and GH production

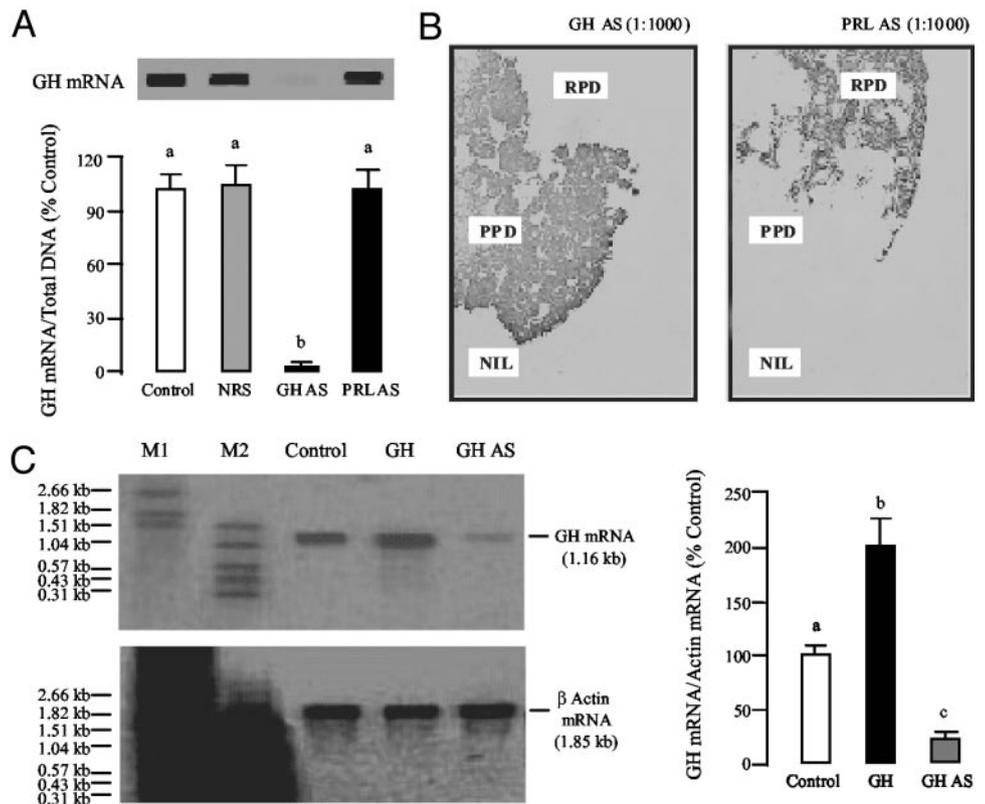
To test for the direct actions of exogenous GH on GH gene expression at the pituitary level, dispersed pituitary cells were incubated for 48 h with increasing doses (3–1000 ng/ml) of porcine GH (Fig. 1A) and sea bream GH (Fig. 1B). In this case, both porcine and sea bream GH was effective in elevating steady-state GH mRNA levels with peak responses noted at 30 and 100 ng/ml, respectively. At higher concentrations of GH tested, the magnitude of GH mRNA responses declined gradually. In parallel experiments, GH production was monitored with a RIA for carp GH after a 48-h incubation with porcine GH. Unlike the carp GH standard, which caused a dose-dependent decrease in the specific binding of ^{125}I -labeled GH tracer, increasing doses of porcine GH up to

1000 ng/ml level could not displace the specific binding of GH tracer in our assay system (Fig. 2A). These results indicate that the RIA used in this study exhibits no cross-reactivity with porcine GH. Parallel to its effect on GH mRNA expression, increasing doses (10–1000 ng/ml) of porcine GH were effective in elevating GH production, and a bell-shape curve was obtained with the peak response noted at 100 ng/ml (Fig. 2B).

GH immunoneutralization on GH mRNA expression

To test whether endogenous GH released from pituitary cells could alter GH mRNA expression, removal of secreted GH in cell cultures was performed by GH immunoneutralization. In this case, a 48-h incubation of pituitary cells with increasing levels (1:10,000 to 1:500) of carp GH antiserum resulted in a dose-dependent decrease in steady-state GH mRNA levels, with an IC_{50} of approximately 1:4000 and maximal inhibitory effect at 1:1500 (Fig. 3A). In parallel experiments, GH antiserum (1:2500) did not affect basal expression of $GTH\alpha$ mRNA levels (Fig. 3A, *small inset*), suggesting that the effect of GH immunoneutralization was specific for GH transcripts. The inhibitory action of GH antiserum (1:1000) was time dependent, and a 20-h treatment was sufficient to induce a maximal inhibition on GH mRNA expression (Fig. 3B). In the time-matched controls, static incubation of pituitary cells up to 50 h did not alter basal GH mRNA expression. The specificity of the effects caused by GH antiserum was also examined by immunoneutralization using normal rabbit serum (NRS) and an antiserum for carp prolactin (26). Unlike GH antiserum (1:1000), which inhibited GH mRNA expression, the same dilution of NRS or prolactin

FIG. 4. Selectivity of GH antiserum for immunoneutralization and Northern blot analysis of GH transcripts expressed in grass carp pituitary cells. A, Effects of GH antiserum (GH AS), PRL antiserum (PRL AS), and NRS on GH mRNA expression. Pituitary cells were incubated with GH AS, PRL AS, and NRS at 1:1000 dilutions for 48 h before total RNA isolation. B, IHC staining of somatotrophs and lactotrophs in carp pituitary sections. Consecutive pituitary sections were immunostained with GH AS and PRL AS, respectively. GH immunoreactivity was identified exclusively in the proximal pars distalis (PPD), whereas PRL immunoreactivity was restricted to the RPD. However, GH and PRL immunoreactivity could not be detected in the NIL. C, Northern blot of GH transcripts. Pituitary cells were incubated with porcine GH (100 ng/ml) or GH antiserum (1:2000) for 48 h. mRNA was isolated and resolved by electrophoresis in 1% gel. Hybridization signals for GH mRNA were detected using a DIG-labeled cDNA probe for grass carp GH. In this study, parallel probing of β -actin mRNA was used as an internal control. GH mRNA data are expressed as mean \pm SEM (n = 4), and treatment groups denoted with different letters represent significant difference at $P < 0.05$.



antiserum did not alter basal GH mRNA levels (Fig. 4A). Using IHC staining of grass carp pituitary sections, GH antiserum (1:1000) was shown to react with somatotrophs located in the proximal pars distalis but not the cells in the rostral pars distalis (RPD) or neurointermediate lobe (NIL). Immunostaining with prolactin antiserum (1:1000) in the consecutive sections, however, could recognize only lactotrophs located in the RPD (Fig. 3B). These results confirmed that the antiserum used for GH immunoneutralization was specific and did not cross-react with other pituitary hormones. Using Northern blot, a single transcript for GH mRNA with the size of approximately 1.16 kb was noted in the control as well as in the groups treated with either porcine GH or GH antiserum (Fig. 4C). Similar to the results of slot blots, porcine GH (100 ng/ml) increased, whereas GH antiserum (1:2000) decreased basal levels of GH mRNA in grass carp pituitary cells. The levels of β -actin mRNA, the internal control of the experiment, was not affected by treatment with GH or GH antiserum.

GH Immunoneutralization on GH release and cellular GH content

Using Western blot analysis, the effects of 48-h incubation with GH antiserum on basal GH release and GH contents were tested. As shown in Fig. 5A, GH antiserum (1:10,000 to 1:1000) reduced the levels of 22-kDa GH immunoreactivity

released into the culture medium in a dose-dependent manner, whereas the amount of GH immunoreactivity in protein extract prepared from carp pituitary cells was significantly elevated. In the control, the level of GH immunoreactivity released into culture medium was much higher than that of pituitary protein extract, suggesting that the cumulative GH release during the 48-h incubation far exceeded the cellular GH content. The situation, however, was reversed after treatment of pituitary cells with high levels of GH antiserum. The inhibitory effects of GH antiserum (1:2500) on GH immunoreactivity released into the culture medium were also time dependent (Fig. 5B). Within the first 2 h of incubation, only a very faint band of GH immunoreactivity could be detected, and no difference was noted between the control *vs.* the treatment group exposed to GH antiserum. After that, the level of GH immunoreactivity released into the culture medium was increased in a time-dependent manner from 24 to 48 h, but this GH response was markedly suppressed by GH immunoneutralization.

Expression of GH receptors in carp somatotrophs

To test whether somatotrophs were the target cells for GH treatment at the pituitary level, RT-PCR of GH receptors was performed in LCM isolated immunoidentified carp somatotrophs (Fig. 6A). Using primers specific for grass carp GH receptor, a 259-bp PCR product was consistently detected in

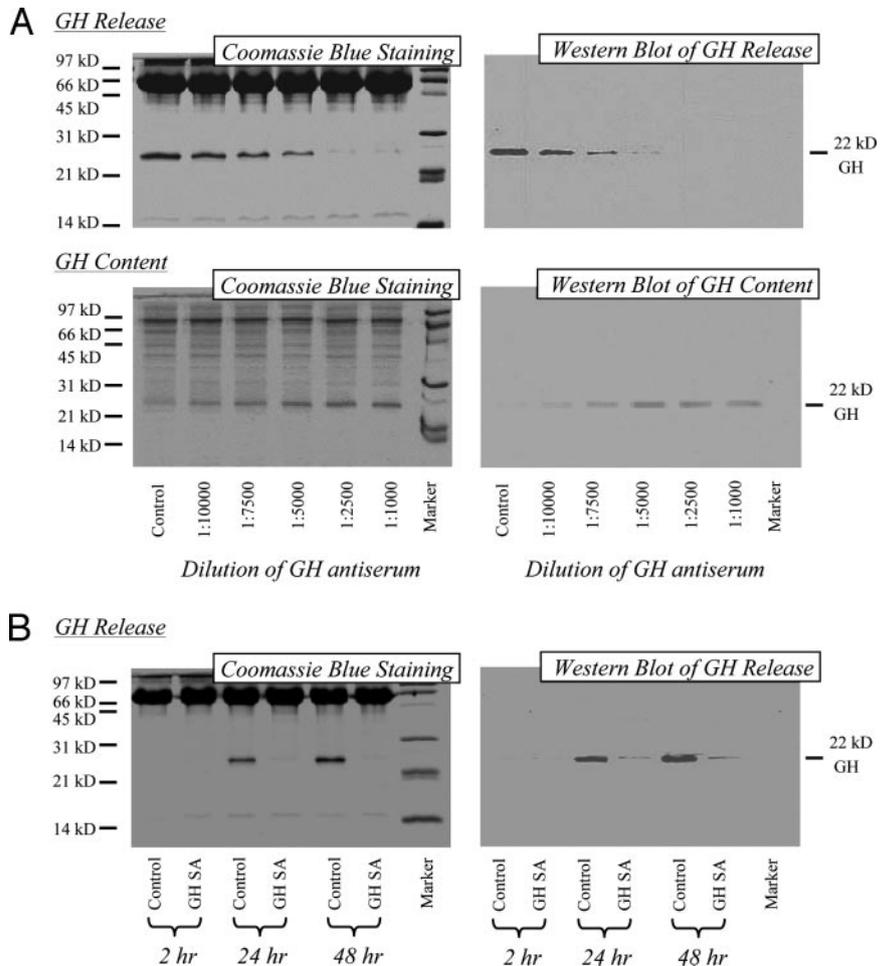
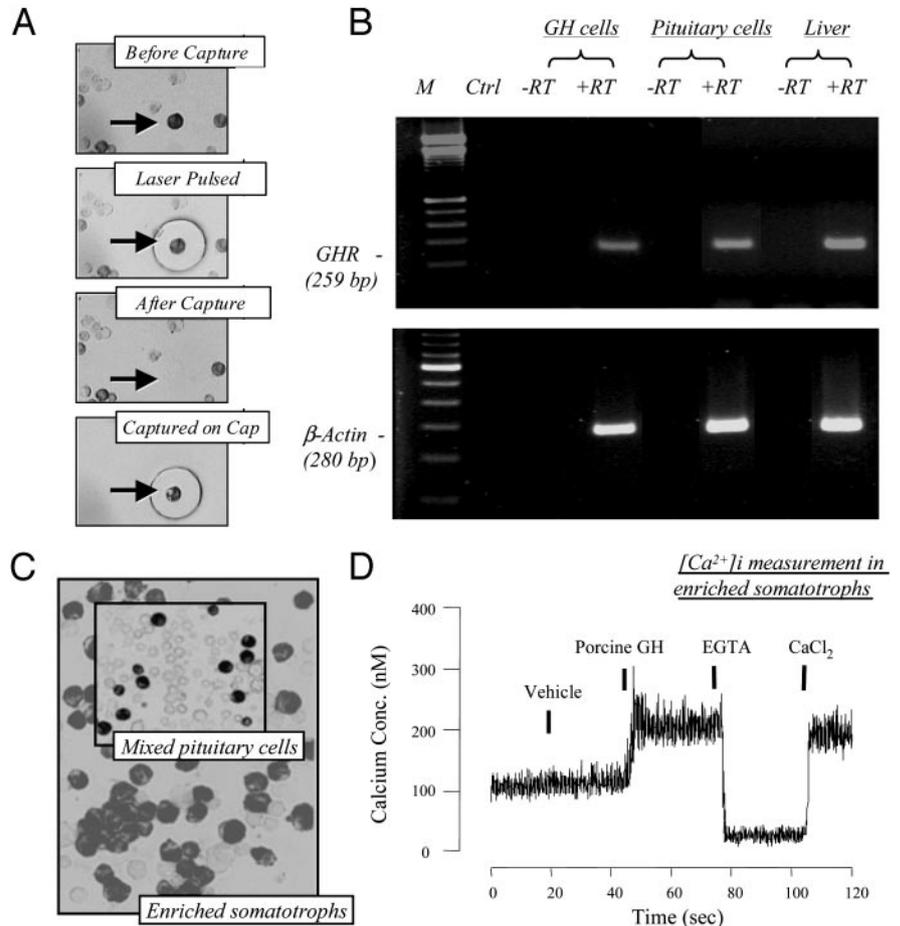


FIG. 5. Effects of GH immunoneutralization on GH release and cellular GH content. A, Dose dependence of GH antiserum treatment on GH release and GH content. Pituitary cells were exposed to increasing levels of GH antiserum for 48 h. Culture medium was harvested for quantitation of GH release. Pituitary cells were lysed and protein extract was prepared for measurement of GH contents. B, Time course of GH antiserum treatment on GH release from grass carp pituitary cells. Pituitary cells were incubated with 1:2500 GH antiserum for the duration as indicated, and culture medium was harvested to monitor changes in GH release. Coomassie blue staining of the protein gels is presented on the right to show equal loading of protein samples in individual lanes, whereas the corresponding Western blot for GH immunoreactivity (22 kDa) is presented on the left.

FIG. 6. Expression of GH receptors in grass carp somatotrophs. **A**, Isolation of immunoidentified somatotroph by LCM. **B**, RT-PCR of GH receptor mRNA in a pure population of grass carp somatotrophs prepared by LCM. Total RNA was isolated from immunoidentified somatotrophs or mixed populations of carp pituitary cells, and RT was performed in the presence (+RT) or absence of Superscript II (–RT). RT-PCR of GH receptor mRNA in the liver was used as a positive control, and PCR of β -actin was used as the internal control. **C**, Enriched somatotrophs prepared from grass carp pituitary cells by density gradient centrifugation. Somatotrophs were identified by immunostaining using GH antiserum (1:8000). Compared with freshly dispersed grass carp pituitary cells (*inset*), a significant enrichment of somatotrophs (~81%) was observed after Percoll gradient purification. **D**, Effects of GH on $[Ca^{2+}]_i$ in enriched somatotrophs prepared from grass carp pituitary cells. Enriched cells were preloaded with the Ca^{2+} -sensitive dye Indo-1/AM and challenged with porcine GH (5 μ g/ml) followed by EGTA (4 mM) and $CaCl_2$ (25 mM).



the RT samples of purified somatotrophs and mixed populations of pituitary cells (Fig. 6B). The 259-bp PCR product for GH receptor was also detected in the liver, which was used as a positive control in these experiments. The authenticity of the PCR product was also confirmed by Southern blot using a DIG-labeled cDNA probe for carp GH receptor (data not shown). Given that activation of GH receptors is known to induce Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels (27), functional expression of GH receptors was also examined by testing the effect of GH on intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). In this case, carp somatotrophs were enriched by density gradient centrifugation (Fig. 6C) and preloaded with the Ca^{2+} -sensitive dye Indo-1/AM (Fig. 6D). The solvent control for GH did not alter basal levels of $[Ca^{2+}]_i$ in enriched somatotrophs, whereas a rapid rise in $[Ca^{2+}]_i$ was noted when the cells were exposed to porcine GH (5 μ g/ml). This Ca^{2+} response could be abolished by removing extracellular Ca^{2+} using EGTA (4 mM) and restored by introducing a saturating dose of $CaCl_2$ (25 mM).

GH immunoneutralization on GH mRNA expression induced by GH-releasing factors

To examine whether GH immunoneutralization could affect the responsiveness of pituitary cells to stimulation by GH secretagogues, GH gene expression induced by GH-releasing factors in fish, including GnRH, dopamine, and

PACAP, were tested in the presence of GH antiserum. In this case, a 48-h incubation with the dopamine agonist apomorphine (1 μ M, Fig. 7A), PACAP (1 μ M, Fig. 7B), and GnRH (1 μ M, Fig. 7C) significantly increased steady-state GH mRNA levels in carp pituitary cells. These stimulatory actions, however, were inhibited in a dose-dependent manner by increasing levels of GH antiserum (1:10,000 to 1:1500). In these experiments, blockade of the effects by these GH-releasing factors was noted at a 1:5000 dilution of GH antiserum, and GH mRNA expression was abolished when GH antiserum was increased to 1:1500 dilution.

Molecular mechanisms for GH-induced GH gene expression

To elucidate the molecular mechanisms for GH-induced GH gene expression, the effect of GH immunoneutralization on GH mRNA degradation was studied in pituitary cells treated with the transcription inhibitor actinomycin D (Fig. 8). The clearance rate of GH transcripts expressed as the time required for half of the original level of GH mRNA to degrade (*i.e.* $T_{1/2}$) was used as an index to monitor GH transcript stability. Incubation of pituitary cells with 1:2500 and 1:1000 dilutions of GH antiserum induced a dose-dependent shift of the GH mRNA clearance curve to the right. As a result, the deduced $T_{1/2}$ values reduced from 45 h in the control to 21 and 15 h for the groups exposed to 1:2500 and 1:1000 dilutions of GH antiserum, respectively.

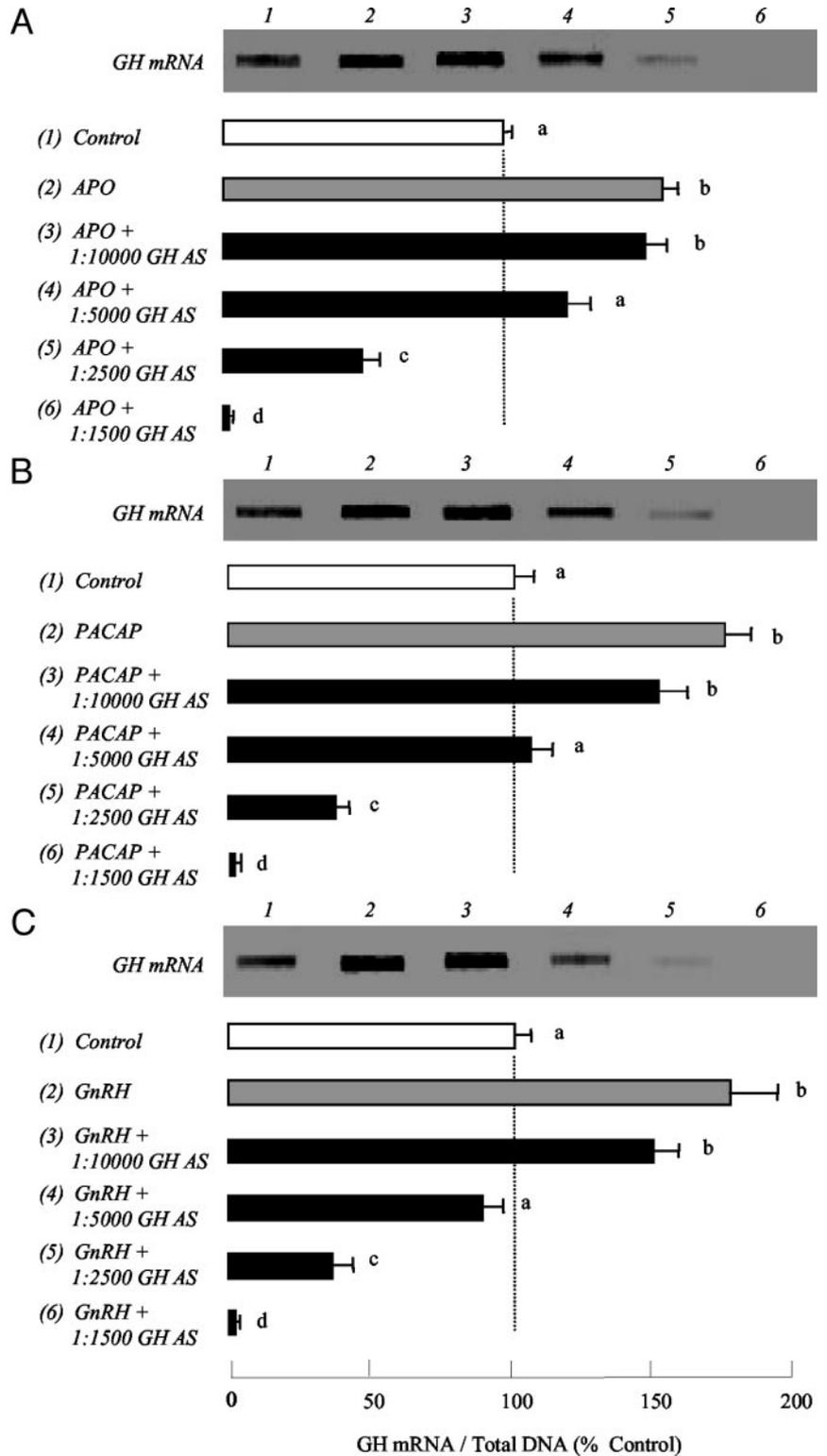


FIG. 7. Effects of GH immunoneutralization on GH mRNA expression induced by GH-releasing factors. Grass carp pituitary cells were incubated for 48 h with the dopamine agonist apomorphine (APO, 1 μ M) (A), ovine PACAP₃₈ (PACAP, 1 μ M) (B), and salmon GnRH (GnRH, 1 μ M) (C) in the presence or absence of increasing levels of GH antiserum. GH mRNA data are expressed as mean \pm SEM (n = 6 wells), and treatment groups denoted with *different letters* represent significant difference at $P < 0.05$. Representative results of slot blot are also included for individual experiments.

To examine the role of GH gene transcription on GH-induced GH mRNA expression, a RPA system was established for simultaneous detection of cytosolic GH mRNA and nuclear GH primary transcript. The riboprobe used in this assay covered the sequence of carp GH gene around the junction between exon II and intron II (position 348–557, Fig.

9A). Using the sense-strand cRNA of the same region as a standard, a dose-dependent rise in the intensity of a 207-bp protected fragment was observed. The signals of this 207-bp fragment increased in a linear fashion with respect to the level of cRNA standard (Fig. 9B). These results indicate that the RPA is suitable for quantitation of nuclear GH primary

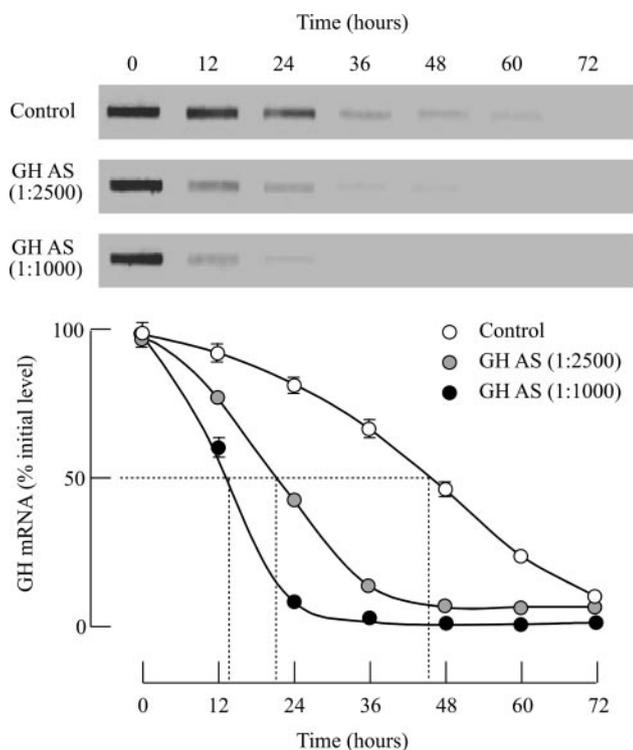


FIG. 8. Effects of GH immunoneutralization on GH mRNA stability. Pituitary cells were incubated with actinomycin D ($8 \mu\text{M}$) in the presence or absence of either 1:2500 or 1:1000 GH antiserum for the duration as indicated. Total RNA was isolated at the respective time points for the construction of mRNA clearance curves. The half-life ($T_{1/2}$) of GH transcript is defined as the time required for GH mRNA levels to drop to 50% of its original values. In this study, the $T_{1/2}$ value of GH mRNA was reduced from 45 h in the control to 21 and 15 h in the treatment groups exposed to 1:2500 and 1:1000 GH antiserum, respectively.

transcript in carp pituitary cells. Similar linearity was also obtained using another sense-strand cRNA containing only the region of exon II (position 348–471). In this case, a 124-bp protected fragment was produced, and this standard curve was used for the calibration of mature GH mRNA levels in the cytosol (data not shown). Using this RPA, it was shown that treatment with GH antiserum (1:1000) abolished basal expression of cytosolic mature GH mRNA (Fig. 9C) and nuclear GH primary transcript (Fig. 9D). In the same experiment, incubation with porcine GH (100 ng/ml) or sea bream GH (30 ng/ml) increased the levels of mature GH mRNA without affecting the expression of GH primary transcript.

The inhibitory actions of GH immunoneutralization on basal expression of mature GH mRNA and GH primary transcripts were further confirmed using real-time PCR. Quantitative PCR of GH primary transcripts and mature mRNA were performed after a 48-h incubation of pituitary cells with increasing levels of GH antiserum (1:10,000 to 1:1000). Calibration of the data for GH primary transcripts was conducted using linear regression of critical threshold values of the standard curve (Fig. 10A). In the same experiment, a separate standard curve was used for data calibration for mature GH mRNA (data not shown). In this study, immunoneutralization with GH antiserum dose-dependently inhibited basal expression of mature GH mRNA (Fig.

10B) and GH primary transcripts (Fig. 10C). A significant reduction of mature GH mRNA levels was noted at a 1:7500 dilution of GH antiserum, whereas a higher concentration of antiserum (1:5000) was required to induce a drop of GH primary transcripts.

Signal transduction mechanisms for GH-induced GH mRNA expression

To examine the postreceptor signaling mechanisms for GH-induced GH gene expression, pituitary cells were exposed to GH for 48 h in the presence or absence of the inhibitors for JAK₂, MAPK, and PI3K (Fig. 11). Treatment with porcine GH (100 ng/ml) alone consistently elevated steady-state GH mRNA levels. This stimulatory action, however, was blocked by the JAK₂ inhibitor AG490 (100 μM , Fig. 11A), P₃₈^{MAPK} inhibitor SB202190 (20 μM , Fig. 11A), P_{42/44}^{MAPK} inhibitor PD98059 (100 μM , Fig. 11B), and PI3K inhibitor wortmannin (100 nM, Fig. 11B). In the same study, AG490 and SB202190 alone also suppressed basal GH mRNA expression, whereas PD98059 and wortmannin were not effective.

Discussion

In mammals, ultrashort feedback by GH at the pituitary level is still controversial, and the idea is supported by some studies but not others. The interpretation of data is further complicated by two technical difficulties, namely cross-reactivity of exogenous GH added to pituitary cell cultures by GH RIA and saturation/activation of pituitary GH receptors by endogenous GH. In this study, autoregulation of GH synthesis by its own secretion was examined in grass carp pituitary cells. The reasons for using grass carp pituitary cells as a cell model are 2-fold: First, mammalian GH is biologically active in fish (15), binds specifically with GH receptors in Cyprinid (carp) species (28), and does not cross-react with the RIA for carp GH. Second, grass carp is one of the few species in fish that has a specific GH antiserum with antibody titer high enough for immunoneutralization (23). This antiserum can be used as a valuable tool to study the effects of removal of endogenous GH both *in vivo* and *in vitro*. Using static incubation of carp pituitary cells, we demonstrated for the first time that exogenous GH increased steady-state GH mRNA levels and GH production directly at the pituitary cell level. In contrast, removal of endogenously secreted GH by immunoneutralization inhibited basal GH secretion and GH mRNA expression. Unlike mammals, GnRH and dopamine are GH-releasing factors in fish (29) and can induce GH mRNA expression in tilapia (30). PACAP, a member of the glucagon/secretin peptide family, has been proposed to be the ancestral form of GHRH (31), and its functional role as a novel GH-releasing factor in fish has been recently confirmed (32). In carp pituitary cells, the dopamine agonist apomorphine, GnRH, and PACAP were all effective in inducing GH mRNA expression, and these stimulatory actions were blocked in a dose-dependent manner by GH antiserum. These results indicate that GH, besides its role as an endocrine hormone, also acts as a novel intrapituitary autocrine/paracrine factor maintaining basal GH synthesis and secretion as well as the sensitivity of somatotrophs to

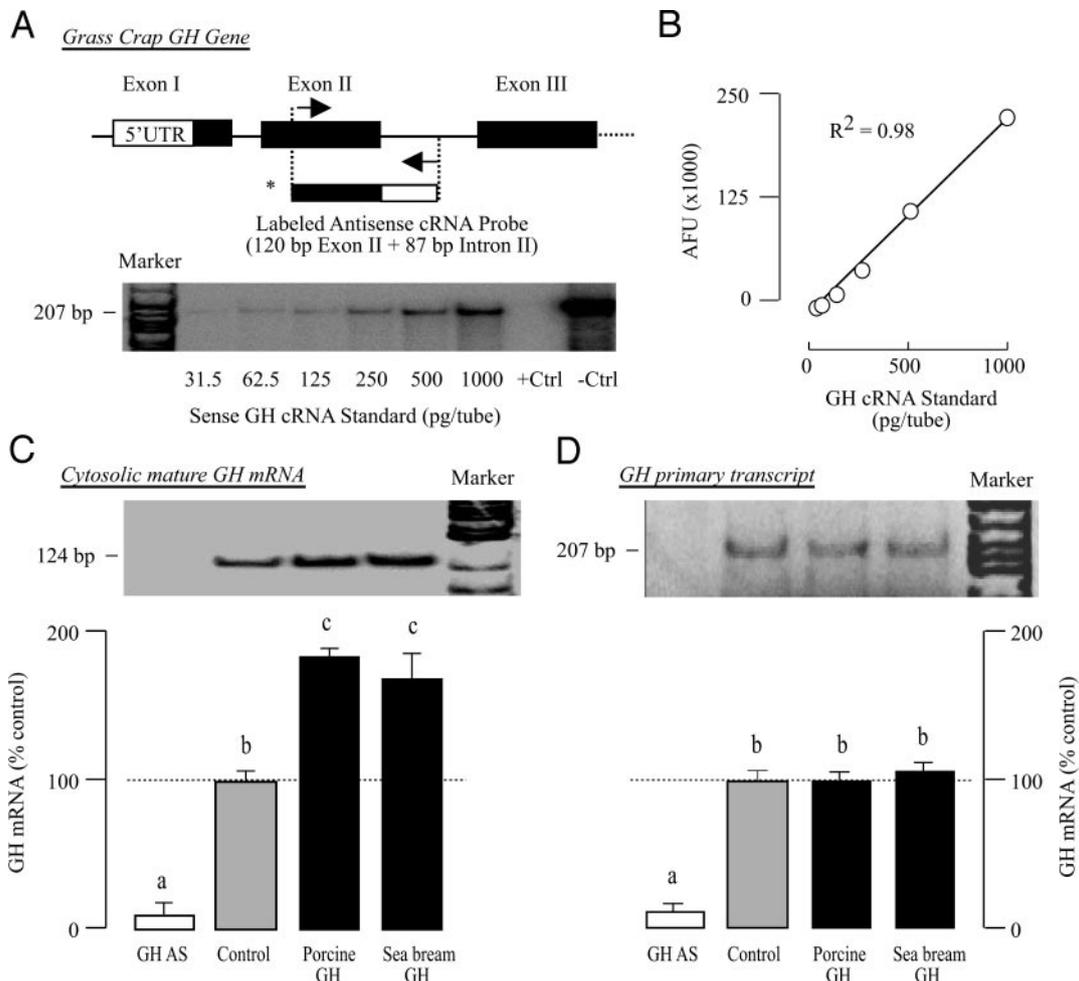


FIG. 9. GH stimulation and GH immunoneutralization on the expression of mature GH mRNA and GH primary transcripts. A, Diagrammatic depiction of the location of antisense riboprobe in grass carp GH gene and the gel picture of the standard curve for GH primary transcripts. Riboprobe alone without RNase digestion was used as a negative control (–Ctrl), whereas a parallel group with pretreatment of RNase A and T1 was used as a positive control (+Ctrl). B, Linear regression of the signal intensity of the standard curve for GH primary transcript as a function of the levels of sense-strand GH cRNA standard. The signal intensity was quantified in arbitrary fluorescence units (AFUs). Cytosolic mature GH mRNA (C) and nuclear GH primary transcripts (D) were monitored in grass carp pituitary cells exposed to 1:1000 GH antiserum (GH AS) to remove endogenous GH or challenged with porcine (100 ng/ml) or sea bream GH (30 ng/ml) for 48 h. GH mRNA data are expressed as mean \pm SEM ($n = 8$ wells), and treatment groups denoted with *different letters* represent significant difference at $P < 0.05$.

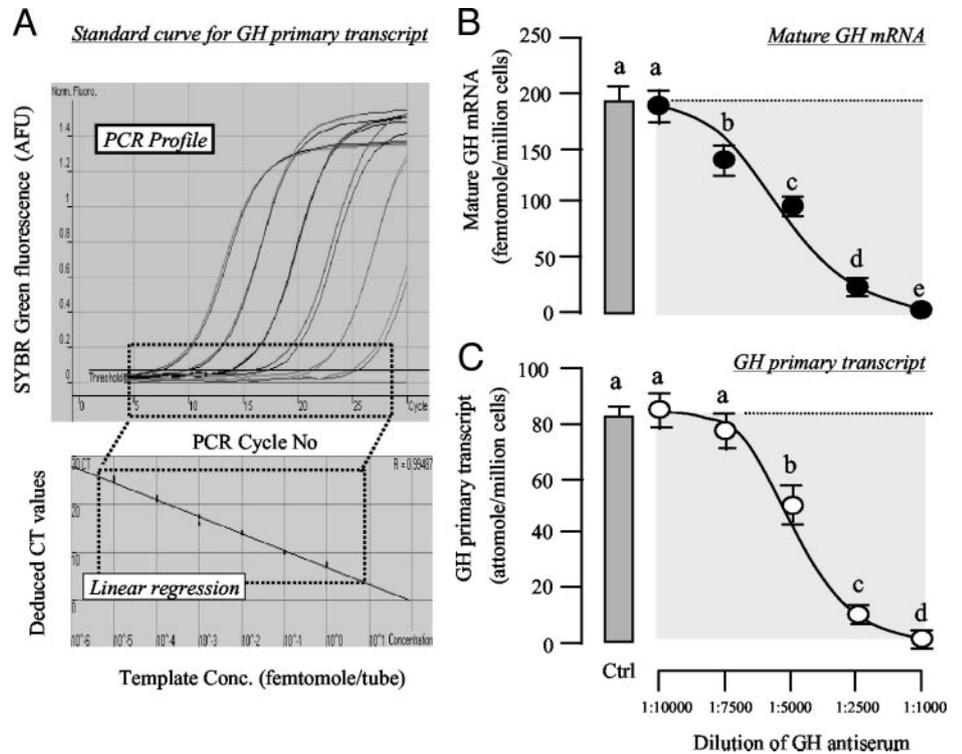
stimulation by GH secretagogues. These findings, however, are at variance with the reports in mammals (11) and argue against the presence of ultrashort feedback at the pituitary level in carp species. This discrepancy may be caused by species-specific variability and/or difference in research methodology.

Recently GH receptors have been cloned in fish, *e.g.* the goldfish (28) and turbot (33), and the receptor mRNA can be detected in the fish pituitary by RT-PCR (28). Using a similar approach, we confirmed the transcript expression of GH receptor in a pure population of grass carp somatotrophs isolated by LCM technique. In parallel experiments, exogenous GH was shown to trigger extracellular Ca^{2+} entry in enriched somatotrophs prepared from carp pituitary cells. In mammals, GH receptors are expressed in the pituitary, *e.g.* in the rat (12), mouse (34), and human (35), and GH receptor activation is known to induce $[Ca^{2+}]_i$ influx via L-type voltage-sensitive Ca^{2+} channels in a protein kinase C-dependent manner (27). Therefore, it is conceivable that functional GH

receptors are expressed in carp somatotrophs and mediate the autocrine/paracrine actions of GH to maintain GH synthesis and secretion.

It is also worth mentioning that cellular GH content was increased in carp pituitary cells after GH immunoneutralization. This observation apparently is contradictory to the idea of GH-induced GH synthesis but may be due to GH accumulation in pituitary cells during the period of suppressed GH exocytosis. Reciprocal relationship of GH secretion and GH contents in pituitary cells has been reported in mammals. For example, bromocriptine, a dopamine agonist, is known to inhibit GH release but increase GH contents in rat pituitary cells (36). In our dose-response studies for GH-induced GH mRNA expression, a bell-shape curve was consistently obtained. A similar dose-response curve has been reported in other GH-mediated responses, *e.g.* GH-induced lipogenesis in rat adipocytes (37). In general, dimerization of GH receptors by sequential interactions with sites 1 and 2 of GH molecule is required for receptor activation (38).

FIG. 10. Dose dependence of GH immunoneutralization on basal expression of cytosolic mature GH mRNA and nuclear GH primary transcripts. A, Standard curve for GH primary transcripts using quantitative real-time PCR. Plasmid DNA containing the full-length grass carp GH gene was used as the standard. The kinetic profiles of PCR amplification with increasing template concentrations are presented in the upper panel. Linear regression of critical threshold (CT) values with respect to template concentrations is presented in the lower panel. Cytosolic mature GH mRNA (B) and nuclear GH primary transcripts (C) were monitored in pituitary cells exposed to increasing levels of GH antiserum for 48 h. GH mRNA data are expressed as mean \pm SEM (n = 8 wells), and treatment groups denoted with different letters represent significant difference at $P < 0.05$.



When GH receptors are exposed to a high dose of GH, a high proportion of receptors will interact with site 1 of GH molecules to form the nonproductive monomeric complex. This will cause a drop in the level of dimeric GH receptors and account for a gradual loss of GH action with increasing GH concentrations (for review, see Ref. 39). Alternatively, the loss of GH responsiveness at high doses could be caused by receptor desensitization. In mammals, GH desensitization has been attributed to degradation of internalized GH receptors (40), cleavage of GH receptors to GH binding protein (38), and GH receptor inactivation by regulators from the CIS/SOCS family (41) or protein tyrosine phosphatases (42). Whether similar mechanisms also exist in fish to modulate GH signaling is unknown and clearly warrants future investigations.

To elucidate the molecular mechanisms for GH-induced GH gene expression, the possible involvement of GH mRNA stability and GH gene transcription in the local actions of GH to regulate its own synthesis were tested. In this case, removal of GH secreted into the culture medium using immunoneutralization caused a drop in the $T_{1/2}$ values of GH mRNA in carp pituitary cells. This result indicates that endogenous GH, by acting in an autocrine/paracrine manner, plays a role in maintaining the stability of GH transcripts, which may represent an important component of GH synthesis in somatotrophs. In the rat, glucocorticoid and thyroid hormone are known to stimulate GH synthesis by increasing $T_{1/2}$ of GH mRNA and these effects are mediated by increasing the length of the poly(A⁺) tail in GH transcripts (43). A similar mechanism, however, may not be applicable to GH-induced GH mRNA expression in carp pituitary cells because the size of GH transcript (~1.16 kb) was not modified by GH treatment or GH immunoneutralization as re-

vealed by the results of Northern blot. Apparently GH autoregulation is also required for the maintenance of GH gene transcription. Our studies on cytosolic mature GH mRNA and nuclear GH primary transcript using real-time PCR and RPA revealed that exogenous GH could elevate the levels of mature GH mRNA in the cytosol, and the accumulation of cytosolic GH mRNA under basal conditions was dependent on endogenous GH. Besides, GH primary transcript in the nuclear fraction was reduced in a dose-dependent manner to an almost undetectable level by GH immunoneutralization. Given that the level of primary transcript is in general taken as an index for active transcription of target genes (44), a loss of GH primary transcript after removing endogenous GH confirms that GH gene transcription is involved in GH-induced GH mRNA expression.

In this study, GH treatment was not effective in altering the levels of GH primary transcript. This lack of GH response may indicate that the sensitivity to GH is different between GH mRNA stability and GH gene transcription. In our real-time PCR study, a higher level of GH antiserum (1:5000) was used to induce a significant drop in GH primary transcripts when compared with that required for inhibiting basal expression of mature GH mRNA (1:7500). These results prompted us to speculate that a lower dose of GH may be required to activate GH gene transcription, and GH secreted from pituitary cells during the incubation period may be good enough to saturate this transcriptional response. This idea is consistent with the findings that locally produced GH can activate GH receptors more effectively than exogenous GH in BRL and MCF-7 cells (45). However, we do not exclude the possibility that the duration of GH treatment optimized for GH mRNA responses may not be appropriate for the detection of changes in GH primary transcript. In other stud-

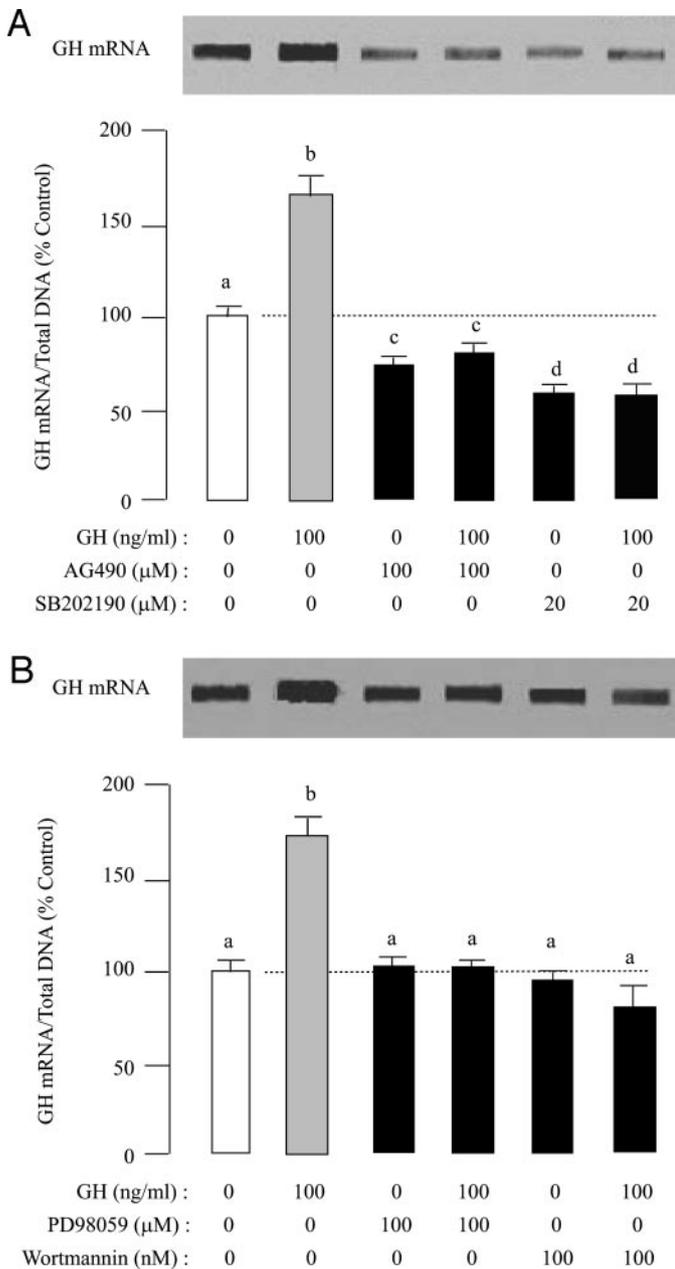


FIG. 11. Functional role of JAK₂, P_{42/44}^{MAPK}, P₃₈^{MAPK}, and PI3K in GH-induced GH mRNA expression in grass carp pituitary cells. A, Pituitary cells were incubated with porcine GH (100 ng/ml) for 48 h in the presence or absence of the JAK₂ inhibitor AG490 (100 μM) and P₃₈^{MAPK} inhibitor SB202190 (20 μM). B, Parallel experiments with the P_{42/44}^{MAPK} inhibitor PD98059 (100 μM) and PI3K inhibitor wortmannin (100 nM). GH mRNA data are expressed as mean ± SEM (n = 8 wells), and treatment groups denoted with *different letters* represent significant difference at *P* < 0.05. Representative results of slot blot are also included for individual experiments.

ies, *e.g.* GnRH-induced LHB and FSHβ gene expression (46), the transcriptional burst of primary transcripts can be rapid and brief (*e.g.* 1 h or less) and precedes the changes in cytosolic mRNA.

In mammals, biological functions of GH is mediated through GH receptor dimerization, recruitment of JAK₂, and subsequent activation of STAT, MAPK, and IRS/PI3K cas-

ades via the assembly of a multiprotein signaling complex (21). JAK₂ is a nonreceptor tyrosine kinase recruited to the membrane proximal proline-rich Box I region of activated GH receptors and ligand-induced phosphorylation of JAK₂ and GH receptors creates the docking sites for STAT binding (47). Activation of GH receptors is known to phosphorylate STAT5a and 5b and increase their binding to γ-interferon-activated site, interferon α-stimulated response element, and/or SIS-inducing element sites in target gene promoters (48). The MAPK cascade has at least three components, namely P_{42/44}^{MAPK} (or ERK_{1/2}), P₃₈^{MAPK}, and c-Jun N-terminal kinase (49), and functional interactions of these components with JAK₂ contribute to the assembly of the multiprotein signaling complex of activated GH receptors (50). This signaling complex is also responsible for phosphorylation of IRS-1 and recruitment of PI3K (51), presumably via focal adhesion kinase/Pky2 association with JAK₂ (50). In this study, the stimulatory action of exogenous GH on GH mRNA expression in carp pituitary cells was abolished by the JAK₂ inhibitor AG490, P_{42/44}^{MAPK} inhibitor PD98059, P₃₈^{MAPK} inhibitor SB202190, and the PI3K inhibitor wortmannin. Besides, basal GH mRNA levels were inhibited by AG490 and SB202190, and PD98059 and wortmannin were not effective in this respect. These results indicate that the JAK₂/MAPK and JAK₂/IRS/PI3K cascades are involved in GH-induced GH gene expression at the pituitary level. Apparently basal levels of GH mRNA expression are maintained by the JAK₂/P₃₈^{MAPK} but not JAK₂/P_{42/44}^{MAPK} and JAK₂/IRS/PI3K cascades.

In summary, using grass carp pituitary cells as a cell model, we have reported for the first time that endogenously secreted GH can serve as an intrapituitary autocrine/paracrine factor maintaining basal GH release, GH gene expression, and somatotroph sensitivity to stimulation by GH-releasing factors. GH-induced GH gene expression is mediated by improving the stability of GH mRNA and activation of GH gene transcription. Apparently the stimulatory action of GH on GH gene expression is dependent on the functional coupling of JAK₂, PI3K, and MAPK to pituitary GH receptors. This intrapituitary GH autoregulation may play a key role in the functionality of the brain-pituitary axis as it can modulate the responsiveness of somatotrophs to hypothalamic factors. In patients with pituitary tumor, hypopituitarism is a common sequela after radiotherapy. Using irradiated rat pituitary cells as a model, GH treatment has been shown to prevent cell death and restore pituitary secretory capacity and responsiveness to GnRH, GHRH, TRH, and CRH, respectively (52). The intrapituitary actions of GH described in this study represent a novel mechanism regulating GH synthesis in somatotrophs and may have clinical implications in cancer treatment.

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